# Gaido lab protocols for digoxigenin-labeled riboprobe synthesis from a PCR-generated DNA template

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## 1. Primer Design

Primers are designed to amplify a 500-1000 bp (750 bp ideal) region of the coding sequence of each gene. Primers are designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) then candidate primer sequences are analyzed using Net Primer (www.premierbiosoft.com/netprimer) to determine the most optimal pair. The reverse primer is linked to a SP6 polymerase promoter tag sequence as follows:

Reverse (antisense) primer: 5' – (leader sequence) GCG – (SP6 sequence) ATTTAGGTGACACTATAG – primer sequence - 3'

Primers are ordered from IDT (Integrated DNA Technologies) and resuspend in RNase-free water to make a 100 μM stock. Make 20 μM working dilutions for each primer, then combine the forward and reverse for each primer set.

Example: 10 μl of the 100 μM Forward primer stock + 10 μl of the 100 μM Reverse primer stock + 30 μl RNase-free water. Store stocks and working dilutions at -20°C.

## 2. PCR

First round PCR:
Set up 25 μl reactions using Qiagen Taq PCR Master Mix Kit (cat # 201443) as follows:

| | 1x | Final concentration |
|---|---|---|
| Taq PCR master mix | 12.5 μl | 2.5 U Taq, 200 μM dNTPs, 1x buffer, 1.5 μM $MgCl_2$ |
| 20 μM F+R primer | 1 μl | 800 nM |
| cDNA template * | 11.5 μl | |
| Total rxn volume | 25 μl | |

*cDNA template is reverse transcribed from a pool of RNA isolated from fetal testes of various gestation ages.

PCR Cycling conditions:

| | | |
|---|---|---|
| 94°C | 2 min | 1 cycle |
| 94°C<br>55°C<br>72°C | 20 sec<br>30 sec<br>1 min | 35 cycles |
| 72°C | 10 min | 1 cycle |

Electrophorese the entire 25μl reaction on a 1% agarose 1xTAE gel. Check for a single band of the right size, cut it out of the gel, and purify using Qiagen's QiaQuick Gel Extraction kit (cat# 28706). Elute purified PCR products with 50μl elution buffer then set up a second PCR using the template generated in the first PCR.

Second Round PCR:
Set up 50μl reactions using Qiagen Taq PCR Master Mix Kit (cat # 201443) then run the same cycling conditions as the first round PCR:

| | 1x | Final concentration |
|---|---|---|
| Taq PCR master mix | 25 μl | 2.5 U Taq, 200 μM dNTPs, 1x buffer, 1.5 μM $MgCl_2$ |
| 20 μM F+R primer | 2 μl | 800 nM |
| Template PCR product | 2 μl | |
| $H_2O$ | 21 μl | |
| **Total rxn volume** | **50 μl** | |

Electrophorese the entire 50μl reaction on a 1% agarose 1xTAE gel. Check for single bands of the right size, cut them out of the gel, and purify using Qiagen's QiaQuick Gel Extraction kit (cat# 28706). Elute purified PCR products with 50μl elution buffer. DNA concentration is measured using 2μl on a NanoDrop and another 2μl is electrophoresed on a 1% agarose 1xTAE gel to verify a single band of appropriate size.

Sequence Verification:
100-200 ng of each PCR product is sent to MWG for sequence verification (ValueRead sequencing using SP6 reverse primer). Once sequences are confirmed, continue with production of DIG-labeled riboprobes.

## 3. *In vitro* Transcription of Digoxigenin-Labeled Riboprobes for *in situ* Hybridization

Roche DIG RNA Labeling Kit (SP6/T7) – cat# 11175025910

Start with 200ng template PCR product, bringing volume to 13μl with RNase-free $H_2O$. Keep samples and the following reagents on ice:

| | 1x |
|---|---|
| 10x NTP labeling mix | 2 μl |
| 10x transcription buffer | 2 μl |
| Protector RNase Inhibitor | 1 μl |
| RNA polymerase SP6 (20U/μl) | 2 μl |
| Template + $H_2O$ | 13 μl |
| **Total rxn volume** | **20 μl** |

Mix briefly and centrifuge.
Incubate at 37°C for 2 hrs.
Add 2μl DNase I and incubate at 37°C for 15 min.
Stop rxn with 2μl 0.2M EDTA (pH 8).
Precipitate by adding 66μl $H_2O$, 10μl 4M LiCl, 300μl 100% EtOH pre-chilled to -20°C. Precipitate at -20°C overnight.
Centrifuge at 13,000 rpm, 4°C, 15 min then wash 2x with 300ml 70% EtOH (13,000 rpm, 4°C, 15 min each time).
Allow pellet to air dry ~15 min then resuspend in 23μl $H_2O$.
DNA concentration is measured using 2μl on a NanoDrop and 1μl is electrophoresed on a 1% agarose 1xTAE gel (should see smear with some bright bands).
Bring concentration of all DIG-labeled riboprobes to 100ng/μl with RNase-free $H_2O$.

## 4. Determination of Labeling Efficiency of DIG-labeled Riboprobes

Roche DIG Nucleic Acid Detection kit - cat# 11175041910

Solutions to be prepared in advance (stable for long-term):

**Maleic acid buffer** (stable at 15-25°C)
- 0.1M Maleic acid (CAS# 110-16-7)
- 0.15M NaCl (CAS# 7647-14-5)
- Adjust pH to 7.5 w/ NaOH pellets (CAS# 1310-73-2)

**Washing buffer** (stable at 15-25°C)
- 0.1M Maleic acid
- 0.15M NaCl
- Adjust pH to 7.5 w/ NaOH pellets
- Add 0.3% (v/v) Tween 20 (CAS# 9005-64-5)

**Detection buffer** (stable at 15-25°C)
- 0.1M Tris-HCl
- 0.1M NaCl
- Adjust to pH 9.5

**10x Blocking reagent** (stable at 2-8°C or -15 to -25°C)
- ~50 g per bottle, comes with kit.
- Dissolve in Maleic acid buffer to a final concentration of 10% (w/v) = ~500ml.
- Requires heating and shaking (stir on heated magnetic stir plate ~20 min).
- Autoclave this stock solution.

Solutions to be prepared fresh each time:

**RNA dilution buffer** (see p.3 of Roche DIG Labeling kit protocol – cat# 11175025910)
- DEPC $H_2O$: 20x SSC: 37% Formaldehyde (CAS# 50-00-0) in the ratio 5:3:2

**Blocking solution** (120 ml per membrane – 100 ml for block, 20 ml for antibody solution)
- Prepare 1x working solution by diluting 10x blocking reagent 1:10 with Maleic acid buffer

**Antibody solution** (20 ml per membrane)
- Centrifuge anti-digoxigenin-AP antibody 5 min at 10,000 rpm prior to use.
- Dilute antibody 1:5000 in blocking solution (4μl in 20ml).

**Color substrate solution** (20 ml per membrane)
- Add 400 ml of NBT/BCIP stock solution to 20 ml detection buffer.
- *Store protected from light.*

Prepare dilution series of the labeled RNA probe and control DNA as described in the table on page 13 of Roche DIG High Prime DNA Labeling and Detection starter kit protocol (cat# 11745832910), version December 2005.

Cut positively charged nylon membrane (Roche, cat# 1209272) to fit in a 200μl pipet tip box then apply a 1μl spot of each dilution on the membrane.

Fix nucleic acid to the membrane by UV-crosslinking at 120mJ in Stratalinker.

Place membrane in the sterile 200μl pipet tip box, then follow the protocol as described in Roche DIG Nucleic Acid Detection kit - cat# 11175041910.

Allow the color development to continue for the full 16 hours then stop the reaction with 50μl DEPC-$H_2O$.

While the membrane is still wet, visually inspect the membrane and record the lowest concentration at which a colored spot is detected for each probe. Wrap membrane in plastic wrap and make a photocopy for records.

100% labeling efficiency is achieved when a spot can be seen at the 0.1 pg/μl spot however, probes that yield a spot at 0.3 pg/μl are still perfectly viable for ISH.

Make several 5μl aliquots of labeled riboprobes and store at -80$^{o}$C, avoid freeze/thawing.